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Identification and Serotyping of *Ornithobacterium rhinotracheale*

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In the present study 443 strains of *Ornithobacterium rhinotracheale*, a causative agent of respiratory disease in fowl, were investigated biochemically and serologically. In both ways *O. rhinotracheale* could be differentiated from other gram-negative rods and, more particularly, from the *Pasteurella*-like bacteria potentially pathogenic for fowl. For the biochemical characterization of *O. rhinotracheale* the API 2ONE identification strip proved to be useful, although *O. rhinotracheale* is not included in the API system. Serologically, by using monovalent antisera in agar gel precipitation (AGP) tests and enzyme-linked immunosorbent assays (ELISAs), seven serotypes (serotypes A to G) of *O. rhinotracheale* could be discriminated. The AGP test was chosen as the preferred method to be used for serotyping. Isolates of serotype A were found to be the most prevalent, especially in chickens. Isolates from turkeys were more heterogeneously divided over the serotypes. Some strains showed cross-reactivity between serotypes A, B, and E. Five *O. rhinotracheale* strains could not be serotyped with the available antisera. Relationships between the geographic origin and the serotypes were found. By the ELISA the presence of antibodies against *O. rhinotracheale* could be detected in 1-day-old birds as well as in birds with clinical signs, and therefore, it might be useful for diagnostic purposes.

Respiratory problems, together with purulent pneumonia, airsacculitis, severe growth retardation, and rapidly increasing mortality, were reported in meat turkeys and broilers in South Africa, Germany, the United States, France, and The Netherlands (1, 2, 4, 6). A gram-negative, pleomorphic rod could repeatedly be isolated from affected organs. This *Pasteurella*-like organism has recently been referred to as *Ornithobacterium rhinotracheale* gen. nov. sp. nov. (7). In experimental infections, it was possible to evoke severe growth retardation and airsacculitis in turkeys and chickens with this bacterium (8). *O. rhinotracheale* strains from different countries reporting fowl infected with the organism were investigated for their bacteriological, biochemical, and serological relationships and their differences from other gram-negative rods.

MATERIALS AND METHODS

Bacterial strains. The 443 *O. rhinotracheale* strains involved in this study were isolated between 1987 and 1996 from the respiratory tract, liver, joints, or brain of diseased birds in various countries. All except three strains were isolated from chickens or turkeys; the other three strains were isolated from a duck (from Hungary) and from a guinea fowl and a partridge (both from France). Immediately after arrival the strains were freeze-dried and stored at −20°C. The strains were cultivated on sheep blood agar at 37°C in a 5 to 10% CO_2 atmosphere for at least 48 h.

For comparative investigations, the 16 serotype-specific strains of *Pasteurella multocida* (3), the 17 serotype-specific strains of *Riemerella anatipestifer* (5), and the 3 serotype-specific strains of *Haemophilus paragallinarum* (9), together with 2 NADH-independent *H. paragallinarum* strains from South Africa (J. du Preez) and a field strain of *Pasteurella gallinarum* (our own collection), were used. Because in the beginning we found *O. rhinotracheale* to react biochemically as a *Kingella*-like bacterium, the strains *Kingella kingae* ATCC 23330, *Kingella denitrificans ATCC 33394, and Kingella* (*Suttonella*) *indologenes* ATCC 25869 were also included in the comparative studies.

Biochemical and enzymatic reactions. The biochemical and enzymatic reactions were tested by using the API system (BioMérieux SA, La Balmes-Les Grottes, France). All tests were performed under the prescribed conditions. As in the identification method used by the API system, *O. rhinotracheale* strains, which are oxidase-positive, gram-negative, facultatively anaerobic rods, were characterized with the use of the API 2ONE strip.

Serological investigations. Monovalent antisera were prepared by injecting specific-pathogen-free chickens subcutaneously twice with a bacterin containing between 10^8 and 10^9 formalin-killed bacteria per dose in an oil adjuvant at an interval of 3 to 4 weeks. At 3 to 4 weeks after the second injection, serum was collected.

Boiled extract antigens (BEAs) were prepared as described by Heddleston (5) by washing well-grown cultures from sheep blood agar with 0.02 M phosphate buffer–8.5% NaCl–0.3% formaldehyde (pH 7.2). The same buffer was used to adjust the suspensions to an optical density at 660 nm of 0.5 to 0.6 when the suspensions were diluted 1:20. Subsequently, the suspensions were boiled for 60 min at 100°C. After centrifugation the supernatants were sterilized by filtration through a 0.22-μm-pore-size filter and were then used as antigen in agar gel precipitation (AGP) tests as well as in enzyme-linked immunosorbent assays (ELISAs).

For the AGP test, glass slides were flooded with ±1 ml of preheated sterile 1.5% Noble agar–8.5% NaCl–0.1% thimerosal per 5 cm^2. Patterns consisting of six or seven wells (2 mm in diameter) located around a central well at a distance of ±5 mm were punched out of the agar. At 1-h intervals the wells were filled twice with ±15 μl of undiluted BEAs or serum. The slides were incubated for at least 48 h in a moist chamber at 37°C and were then observed for precipitation lines under UV light.

For the ELISA, polystyrene microtitre plates were coated with a 1/100 dilution of BEAs and were incubated overnight at 37°C. The coated plates were incubated for 60 min at 37°C with serial dilutions of the test sera. Subsequently, the bound antibodies were quantified with rabbit anti-chicken immunoglobulin peroxidase conjugate (Nordic) and tetramethylbenzidine (Fluka) as the substrate.

RESULTS

Bacteriological identification. Optimal growth of *O. rhinotracheale* was obtained when the organism was incubated on 5% sheep blood agar for at least 48 h under microaerobic conditions (5 to 10% CO_2) at 37°C. Under these circumstances *O. rhinotracheale* developed small grey to grey-white colonies, sometimes with a reddish glow and always with a distinct odor, similar to the odor of butyric acid. Upon primary isolation, most *O. rhinotracheale* cultures showed great differences in colony size, from 1 to 3 mm after 48 h of incubation. When the primary cultures were subcultured, the colony size became more uniform.

The most important biochemical and enzymatic reactions of *O. rhinotracheale* in comparison to those of *Kingella* spp. and the gram-negative rods related to the family *Pasteurellaceae* potentially pathogenic for fowl are listed in Table 1. By using the same tests and references, *O. rhinotracheale* can also be differentiated from other potentially pathogenic gram-negative rods in the families *Enterobacteriaceae* and *Neisseriaceae*.

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TABLE 1. Differentiation of *O. rhinotracheale* from *Kingella* and gram-negative rods related to *Pasteurellaceae* potentially pathogenic for fowl[a]

Reaction	Result								
	Haemophilus paragallinarum[b]	*Riemerella anatipestifer*[b]	*Pasteurella multocida*[b]	*Pasteurella haemolytica*[b]	*Pasteurella gallinarum*[b]	*Yersinia* (*Pasteurella*) *pseudo-tuberculosis*[b]	*Actinobacillus* spp.[b]	*Kingella* spp.[b]	*Ornithobacterium rhinotracheale*
Nitrate reduction	+[c]	−	+	+	+	+/−		+/−	−
Catalase	−	+	+	+	+	+	+	−	−
Oxidase (cytochrome)	−	+	+	+	+	−	+	+	+
Urease	−	+/−	−	−	−	+	+/−	−	+/−
β-Galactosidase (ONPG[d])	+	−	+/−	−	+/−	+	+/−	−	+
ADH	−	+	−		−		−	−	−/+
Indole	−	+/−	+	−	−	−	−	+/−	−
Growth on MacConkey agar	−	−	−	−	−	+/−	+		−
Lysine decarboxylase	−		−		−	−	−	−	−
Ornithine decarboxylase	−		−		−	−	−	−	−
Fermentation or oxidation of:									
Fructose	+	−			+	+		−/+	+
Lactose	−	−	−	+/−	−	−	+/−	−	+
Maltose	+	−	+/−	+	+	+	+	+/−	+
Galactose	−	−	+		+	+	+/−	−	+

[a] Data are from *Diseases of Poultry*, 9th ed. (9).
[b] Data on biochemical reactions are from *Bergey's Manual of Determinative Bacteriology*, 9th ed. (3a), and the analytical profile index of the API identification system.
[c] +, positive; +/−, majority of strains positive; −/+, majority of strains negative; −, negative.
[d] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

Within the species *O. rhinotracheale*, the enzymatic reactions (performed with API ZYM strips) showed uniform results.

All 443 *O. rhinotracheale* strains reacted positively in the oxidase test. When tested in the API 2ONE strip at the recommended temperature of 30°C, the *p*-nitrophenyl-β-D-galactopyranoside test (to observe the presence of β-galactosidase) became positive within 3 h for all strains. With the API 2ONE strip, 65% of the strains also reacted positively in the urease test and four strains (1%) reacted positively in the arginine dihydrolase (ADH) test. For all other tests used in the API 2ONE strip, no reactions were seen even after 72 h of incubation at 30°C. When strains were tested at 37°C in the API 2OE strip, the urease, ADH, and gelatin (for the presence of protease) tests sometimes reacted positively within 48 h, even if they were negative at 30°C by the API 2ONE test.

AGP test. By using BEAs in the AGP test, seven different serotypes (serotypes A to G) could be distinguished (Table 2 and Fig. 1). Within serotypes A, B, D, and E, different precip-

TABLE 2. Serotyping of *O. rhinotracheale* by AGP test and differentiation of *O. rhinotracheale* from other relevant gram-negative rods by ELISA

Strain no.	Monovalent antiserum			ELISA titer (2 log) against antigens of strain no.:							
	Species	Strain	Serotype	1	2	3	4	5	6	7	Homologous
1	*O. rhinotracheale*	B 3263/91	A	20	15	8	12	12	11	13	20
2	*O. rhinotracheale*	GGD 1261	B	13	19	8	11	13	11	11	19
3	*O. rhinotracheale*	ORV K91-201	C	9	10	17	7	9	11	11	17
4	*O. rhinotracheale*	ORV 94108 no. 2	D	10	11	10	19	11	11	12	19
5	*O. rhinotracheale*	O-95029 no. 12229	E	13	16	11	12	20	11	13	20
6	*O. rhinotracheale*	ORV 94084 K858	F	10	11	11	8	8	20	9	20
7	*O. rhinotracheale*	O-95029 no. 16279	G	11	12	12	9	10	11	20	20
8	*P. multocida*	X-73	1	<6	<6	<6	—[a]	—	—	—	22
9	*P. multocida*	P-1059	2	<6	<6	<6	—	—	—	—	21
10	*P. multocida*	P-1662	3	<6	<6	<6	—	—	—	—	20
11	*P. multocida*	P-1702	4	<6	<6	<6	—	—	—	—	19
12	*R. anatipestifer*	PAA CV	1A	7	<6	<6	—	—	—	—	18
13	*R. anatipestifer*	PAB BRD	6B	<6	7	7	—	—	—	—	20
14	*R. anatipestifer*	PAD CV	10D	<6	<6	<6	—	—	—	—	21
15	*H. paragallinarum*	0083	A	8	8	8	—	—	—	—	16
16	*H. paragallinarum*	Spross	B	8	8	7	—	—	—	—	18
17	*H. paragallinarum*	H-18	C	9	8	8	—	—	—	—	16
18	*H. paragallinarum*[b]	281/91	A	10	8	7	—	—	—	—	16
19	*H. paragallinarum*[b]	4620/91	A	8	8	7	—	—	—	—	12
20	*P. gallinarum*	Field strain		8	6	7	—	—	—	—	17
21	*K. kingae*	ATCC 23330		9	7	8	—	—	—	—	18
22	*K. denitrificans*	ATCC 33394		7	7	7	—	—	—	—	19
23	*K. indologenes*	ATCC 25869		8	6	7	—	—	—	—	17

[a] —, not determined.
[b] NADH-independent strain.

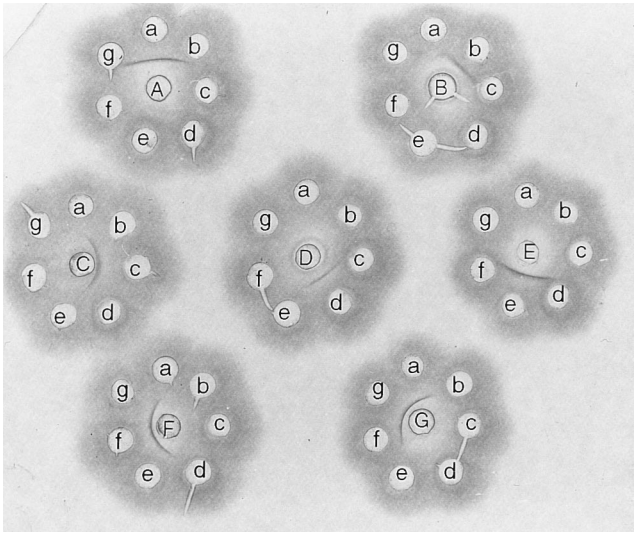

FIG. 1. Differentiation of the seven serotypes of *O. rhinotracheale* (serotypes A to G) by the AGP test. Capital letters, antigens; lowercase letters, monovalent antiserum.

itation reactions were seen. The reactions of 88% of the strains belonging to serotypes A, B, D, or E were visualized as sharp precipitation lines. When BEAs of different strains within a serotype were tested against the corresponding antiserum, the precipitation patterns of the sharp lines showed that the precipitating antigens were identical. The BEAs of the remaining 12% of the strains produced faint precipitation lines that also appeared to represent identical precipitating antigens within each serotype, as indicated by the precipitation pattern that was found. The BEAs of eight strains were found to react with more than one antiserum. In addition to a sharp precipitation line against serotype A or B antiserum with these strains, several faint precipitation lines against serotype A, B, or E antiserum were seen. Five strains (1%) could not be typed by the AGP test with the presently available antisera. All four strains which reacted positively in the ADH test with the API 2ONE identification strip at 30°C were positive by the AGP test, indicating that they do belong to the species *O. rhinotracheale*. Information about the geographic origin in relation to the serotypes of 440 *O. rhinotracheale* strains isolated from chickens and turkeys is listed in the Tables 3 and 4, respectively.

No reactions were seen in the AGP test between the antigens (BEAs) of the 45 tested *O. rhinotracheale* strains and the antisera against the *Kingella* strains or the *P. gallinarum* strain. Also, no reactions were seen between the antigens of the 45 tested *O. rhinotracheale* strains and all the serotype-specific antisera of 3 *Pasteurella*-like rods (*P. multocida*, *R. anatipestifer*, and *H. paragallinarum*) known to be pathogenic for fowl.

ELISA. By using the ELISA and monovalent antisera (Table 2), the *O. rhinotracheale* strains could be serotyped as described above for the AGP test. Also with the use of this ELISA, *O. rhinotracheale* strains could be distinguished from other relevant gram-negative rods potentially pathogenic for fowl and with which *O. rhinotracheale* could be confused. The monovalent antisera contained large amounts of homologous antibodies (2 log titers up to 22), meaning that background reactions can easily occur. So, with these sera, we regard ELISA titers up to 10 (2 log) to be a negative reaction, which is an arbitrary cutoff. In addition to the data presented in Table 2, antisera raised against the *O. rhinotracheale* serotypes did not react in ELISAs with antigens prepared from the other species listed. Within each species cross-reactions occurred between the serotypes, but few cross-reactions between the species were seen. All antisera showed the highest titer against the homologous antigen. Within the species *O. rhinotracheale*, the cross-reactions were mainly between serotypes A, B, D, and E.

In day-old turkeys as well as day-old broilers, antibodies against *O. rhinotracheale* could be detected. Antibody titers between 8 and 12 (2 log) were found at that time. At 3 weeks of age, the same birds were negative by the ELISA (titers, <5), suggesting that these antibodies were maternally derived. Broiler and turkey flocks from The Netherlands, Germany, France, the United States, and South Africa presenting with clinical signs of an *O. rhinotracheale* infection were investigated for the presence of antibodies by the ELISA. In all cases, antibodies, especially against serotypes A and B, could be found (titers, between 8 and 16).

TABLE 3. Serotypes and geographic origins of the isolates of *O. rhinotracheale* from chickens

Country	No. of strains of serotype:								Total
	A	B	C	D	E	F	G	NT[a]	
France	3				1		1	1	6
Germany	4								4
Italy	1								1
South Africa	57								57
The Netherlands	148	6			3			2	159
United States	19		2						21
Total	232	6	2	0	4	0	1	3	248
Percent	94	2	1	0	2	0	0	1	100

[a] NT, not typeable with the presently available antisera.

DISCUSSION

O. rhinotracheale could be distinguished from other gram-negative rods potentially pathogenic for fowl biochemically as well as serologically. Within the API system *O. rhinotracheale* could easily be identified by the API 2ONE strip at 30°C. Almost all strains (99%) showed an API 2ONE code of 0-2-2-0-0-0-4 (65%) or 0-0-2-0-0-0-4 (34%). If the possibility of a positive ADH test is included (code 0-3-2-0-0-0-4 or 0-1-2-0-0-0-4), a 100% identification score with the API 2ONE strip was found. The enzymes urease, protease, and ADH of *O.*

TABLE 4. Serotypes and geographic origins of the isolates of *O. rhinotracheale* from turkeys

Country	No. of strains of serotype:								Total
	A	B	C	D	E	F	G	NT[a]	
France	35	8		7	12				62
Germany	1	9			1				11
Israel		2							2
The Netherlands	47	22		9	4	1		1	84
United Kingdom	12							1	13
United States	14	3	3						20
Total	109	44	3	16	17	1	0	2	192
Percent	57	23	2	8	9	1	0	1	100

[a] NT, not typeable with the presently available antisera.

rhinotracheale showed temperature-dependent reactions in the API 2OE and API 2ONE strips.

By the AGP test as well as the ELISA, seven serotypes of *O. rhinotracheale* could be distinguished. Some cross-reactions were found between strains of serotypes A, B, D, and E in both tests. In the AGP test it was found that two different precipitating antigens can occur within serotypes A, B, and E, which probably explains why these serotypes also show cross-reactions in the ELISA. These reactions, as well as the five strains (1%) with *O. rhinotracheale*-positive API 2ONE codes which did not react by the AGP test, need to be investigated more thoroughly.

For the identification of *O. rhinotracheale*, we propose the use of the API 2ONE strip at the recommended temperature of 30°C. Strains with the four possible API 2ONE result codes should be further investigated by the AGP test. We recommend the use of the AGP test for serotyping, whereas the ELISA might be useful for diagnosing infections in infected birds. For optimal results the antigens or antisera of all seven serotypes should be used. Therefore, we plan to deposit the serotype-specific *O. rhinotracheale* strains, as listed in Table 2, in the American Type Culture Collection (Rockville, Md.) as reference strains for general use. The serotype specificity can be seen as a disadvantage for the use of the ELISA for diagnostic screening purposes because seven tests must be performed with each serum sample to exclude an infection. We also found that not all the birds within an infected flock were serologically positive and that serological responses in birds infected in the field were sometimes low and could disappear after several weeks. These findings should be kept in mind to prevent incorrect interpretations of ELISA results. More serological data from experimentally and naturally infected birds are necessary to judge the true value of ELISA for diagnostic purposes. Such studies are in progress.

As shown in Tables 3 and 4, serotype A was the predominant serotype among the isolates of *O. rhinotracheale* from chickens and was also the serotype found most frequently among the isolates from turkeys. All strains of serotypes D and F and most strains of serotypes B (88%) and E (77%) in this study were of turkey origin. It is obvious that in this study the distribution of isolates of *O. rhinotracheale* from turkeys among the seven serotypes was more heterogeneous than was the case for the isolates from chickens. Also, relationships between the geographic origins of the strains and the serotypes seem to exist; e.g., all strains of serotype C originated from California, and all strains received from South Africa and the United Kingdom (except for one nontypeable strain) belonged to serotype A. By testing sera from chicken and turkey flocks suspected of being infected with *O. rhinotracheale* by the ELISA, serotypes A and B were found to react the strongest, which is in agreement with the prevalence of these serotypes among the isolated strains. However, it should be kept in mind that the majority of the strains and the sera tested originated from only three areas (France, The Netherlands, and South Africa), which could bias the results.

It is not yet possible to explain the observed differences in the distributions of serotypes among isolates of *O. rhinotracheale* from chickens and turkeys. Previously, we have shown that a serotype A strain from a chicken and a serotype B strain from a turkey have similar virulences for both chickens and turkeys (8). Strains of serotypes C, D, and E are also pathogenic for chickens as well as turkeys (unpublished data). So, there is no indication of any host specificity of the serotypes. A possible explanation may perhaps be found in the different breeding practices used in the chicken and turkey industries, but more thorough epidemiological and pathogenicity studies are needed to obtain final answers to the remaining questions about the emerging threat of *O. rhinotracheale* infections in poultry.

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REFERENCES

1. **Charlton, B., S. Channings-Santagio, A. Bickford, C. Cardona, R. Chin, G. Cooper, R. Droual, J. Jeffrey, C. Meteyer, H. Shivaprasad, and R. Walker.** 1993. Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. J. Vet. Diagn. Invest. **5:**47–51.
2. **Hafez, H. M., W. Kruse, J. Emele, and R. Sting.** 1993. Eine Atemwegsinfektion bei Mastputen durch Pasteurella-ähnliche Erreger: Klinik, Diagnostik und Therapie, p. 105–112. *In* Proceedings of the International Conference on Poultry Diseases.
3. **Heddleston, K. L., J. E. Gallagher, and P. A. Rebers.** 1972. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. Avian Dis. **16:**925–936.

3a. **Holt, J. G., et al. (ed.).** 1994. Bergey's manual of determinative bacteriology, 9th rev. ed. The Williams & Wilkins Co., Baltimore, Md.

4. **Leorat, J., J. Dudouyt, C. Doré, and Y. Gardin.** 1994. *Ornithobacterium rhinotracheale*: une nouvelle raison de tousser. Filières Avicoles **559:**69–70.
5. **Sandhu, T., and M. Leister.** 1994. Serotypes of "*Pasteurella*" *anatipestifer* isolates from poultry in different countries. Avian Pathol. **20:**233–239.
6. **van Beek, P., P. van Empel, G. van den Bosch, P. Storm, P, J. Bongers, and J. duPreez.** 1994. Ademhalingsproblemen, groeivertraging en gewrichtsontsteking bij kalkoenen en vleeskuikens door een Pasteurella-achtige bacterie: *Ornithobacterium rhinotracheale* of "Taxon 28." Tijdschr. Diergeneesk. **119:** 99–101.
7. **Vandamme, P., P. Segers, M. Vancaneyt, K. van Hover, R. Mutters, J. Hommez, F. Dewirst, B. Paster, K. Kersters, E. Falsen, L. Devrieze, M. Bisgaard, K.-H. Hinz, and W. Mannheim.** 1994. Description of *Ornithobacterium rhinotracheale* gen. nov. sp. nov. isolated from the avian respiratory tract. Int. J. Syst. Bacteriol. **44:**24–37.
8. **van Empel, P., H. van den Bosch, D. Goovaerts, and P. Storm.** Experimental infection in turkeys and chickens with *Ornithobacterium rhinotracheale*. Avian Dis., in press.
9. **Yamamoto, R.** 1991. Infectious coryza, p. 186–195. *In* Diseases of poultry, 9th ed. Iowa State University Press, Ames.